Age-Related Changes in Lipid Secretion of Perfused Livers from Male Wistar Rats Donors[1]

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Male Wistar rats show typical age-related variations in the distribution of high-density lipoprotein subfractions that include an increase in HDL1 and a decrease in HDL2 proportion. The role of liver in these variations was evaluated by studying the lipoprotein and bile secretions from perfused livers of 14±1 and 3.5±0.5 month old Wistar rats (adult and young animals, respectively). The lipid content of lipoproteins secreted from adult livers was higher in HDL2 fraction and lower in VLDL fraction. The lipid output did not show significant age-related variations in the case of HDL1 fraction. However, the lipoproteins secreted from adult livers contained a higher proportion of phospholipids, and a lower proportion of triacylglycerols in comparison with lipoproteins secreted by young livers. Therefore, the molar ratio of core to surface lipids was lower in lipoproteins secreted by adult livers. Adult livers showed a reduction in bile flow by about 37% with a significantly higher phospholipid secretion. These findings suggest that both the hepatic metabolism of glycerophospholipids and their repartition between plasma and bile compartments are affected by aging process. In conclusion, present data show that the age-related increase in plasma HDL1 proportion, previously observed in this rat strain *in vivo*, are not due to a higher liver secretion of these particles. Conversely, liver appears to have a major role in the age-related VLDL increase and in the variations of phospholipid lipoprotein secretion.

Key words: aging, lipoprotein secretion, perfused liver, phospholipid, triacylglycerol.

In a previous study we reported a 167% increase in rat plasma total cholesterol levels during the 3 months to 13 months age period (*1*). In the same interval of age, total plasma HDL1 concentration rises from 84 to 149 mg/dl and that of HDL2 and HDL3 decrease from 200 to 166 and from 90 to 70 mg/dl, respectively. As a peculiar age-related variation, we found in fact, that the plasma lipoprotein distribution showed an age-related significant increase in the proportion of HDL1 accompanied with a proportional decrease in both HDL2 and HDL3 fractions, while the proportion of VLDL and LDL were not significantly changed (*2*). The main chemical changes in plasma lipoprotein composition were the age-related increase in cholesteryl ester proportion in all lipoprotein fractions, except HDL2, and the strong decrease in triacylglycerol proportion in VLDL. Liver is likely to be involved in this process, given its major role in lipoprotein metabolism and in regulation of lipid metabolism (*3*). This study tries to clarify the role of liver in age-related changes in circulating lipoprotein by using perfused livers from adult and young rats (14±1 and 3.5±0.5 month old animals, respectively). The choice of these ages as representative of different aging situations was based on our previous finding that the typical lipoprotein profile of old animals started to appear in about 12 month old Wistar rats (*1, 2, 4*). We did not use much older animals to avoid the risk of introducing confounding pathologies and differences in liver viability (*5*).

Results of present study show that liver lipoprotein secretion gives a major contribution to the age-related changes in the plasma lipoprotein profile of Wistar rat strain, but does not explain the increased proportion of cholesterol carried by HDL1 subfractions (*1*). Furthermore, they evidence the relevant role of liver in the age-related changes in the secretion, and probably the synthesis, of lipid classes (in particular the phospholipids) at both sinusoidal and canalicular poles of hepatocyte.

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Abbreviations: Apo, apolipoprotein; CE, cholesteryl ester; EM, electron microscopy; HDL3, high density lipoprotein; HDL1, high density lipoprotein 1; HDL2, high density lipoprotein 2; PL, phospholipid; TG, triacylglycerol; VLDL, very light density lipoprotein.

MATERIALS AND METHODS

All the chemicals and solvents were of analytical grade (Farmitalia Carlo Erba, Milano, Italy). Enzymatic kits for the assay of triacylglycerols, free and total cholesterol were purchased from Boehringer Mannheim Italia (Milano, Italy). 3-α-Hydroxysteroid-dehydrogenase was purchased from Sigma-Aldrich (Milano, Italy). Adult and young male

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Wistar rats (aged 14±1 and 3.5±0.5 months, and weight of 540±37 and 300±45 g, respectively) were purchased from Charles River (Calco-Como Italy). The animals were subjected to a 12 h light cycle and were allowed free access to water and commercial rodent diet ("Dieta Standard" by Mucedola Srl, Italy) for at least two weeks before the study. Both animals and diets suppliers were the same as in previous study (*1*).

Experimental Protocol—Recirculating perfusions were performed with livers of either young or adult rat donors. The bile produced and the lipoproteins secreted into the perfusion medium were collected and quickly processed. The liver microsome membranes were isolated as described by Erickson *et al.* (*6*). The molar ratio of cholesterol to phospholipid in this intracellular membrane was evaluated after each perfusion experiment.

Liver Perfusion—Rats were anaesthetized with Farmotal (Grison Pharma, Rome). The surgical procedure and liver perfusions were performed as previously described (*7, 8*). After a 5 min wash-out with Krebs-Ringer bicarbonate buffer (pH 7.4), livers (weight 16.9±2.9 and 11.9±0.7 g in adult and young rats, respectively) were perfused for 2 h through a recirculating system with a medium volume 5 times higher than the liver weight. The medium contained 1/3 freshly prepared bovine erythrocytes and 2/3 Krebs-Ringer bicarbonate buffer (pH 7.4) together with 4% bovine serum albumin (BSA), 0.1% glucose, and it was recirculated at a flow rate of 1 ml/min per g liver. The perfusion apparatus (Disa, Milan, Italy) was kept at 37°C throughout the experiment. The viability of each liver in the course of perfusion experiments was demonstrated by standard parameters, *i.e.*, aspartate amino-transferase level, O_2 utilization, pH values, and degree of hemolysis (*7*). These parameters did not show significant differences between livers from adult and young rat donors. The volume of bile produced during the perfusion was measured by its weight, assuming a density of 1 g/ml. Its lipid composition was analyzed as described below.

Lipoprotein Isolation—Perfusion medium was collected and immediately centrifuged at 600×*g* for 15 min at 4°C to remove the erythrocytes. Then, the medium was brought to a final density of 1.225 g/ml with NaBr and centrifuged for 24 h at 110,000×*g* (4°C) in a 60 Ti rotor with a Beckman ultracentrifuge. The bottom contained negligible amount of lipids and was discorded. Three milliliters of the total lipoprotein suspension, collected as a floating lipid layer, was used for the separation of lipoprotein fractions by discontinuous gradient ultracentrifugation, as described by Chapman *et al.* (*9*). After centrifugation, the tube was placed in an apparatus for gradient fractionation produced by Hoefer Scientific Instruments (San Francisco, CA). The gradient passed through the flow cell of a UV detector set a 280 nm (Monitor UV-M; Pharmacia Uppsala, Sweden) before it was collected in preweighed tubes with a fraction collector. Single fractions were pooled in four lipoprotein classes according to both their density and protein profile as previously described (*10*). The density ranges of nascent lipoproteins were: VLDL: ≤1.04; HDL1: 1.05-1.09; and HDL2: 1.10-1.21 and a fourth fraction with density higher than 1.21 g/ml. These density ranges of nascent lipoproteins resulted to be only slightly different from plasma lipoprotein fractions isolated with the same analytical procedures (HDL1: 1.05-1.085; HDL2: 1.085-1.125; and HDL3: 1.025-1.25) (*10*). Each lipoprotein pool was extensively dialyzed overnight at 4°C against 0.15 M NaCl, 1 mM EDTA solution (pH 7.4). The dialyzed fractions were submitted to the determination of free and total cholesterol, triacylglycerols, lipid phosphorous, and protein content. Each fraction was also observed by transmission electron microscopy (TEM), and analyzed by native non denaturating polyacrylamide gel disc-electrophoresis (PAGE) as described by Naito *et al.* (*11*). The apolipoprotein profile of each pool was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with the PhastSystem apparatus (Pharmacia, Sweden), following the delipidation of sample, as previously described (*10, 12*).

Analytical Procedures—Each lipoprotein fraction from single perfusion experiment was assayed for protein, free and total cholesterol, triacylglycerol (TG), and phospholipids (PL), thereafter the same lipoprotein fraction isolated from 2 or 3 different perfusions were pooled before the lipid extraction.

Proteins were determined by Bradford's method (*13*), using bovine serum albumin as standard. Free cholesterol (FCH), total cholesterol and triacylglycerols were estimated with commercial enzymatic kits. The lipid phosphorous was determined by colorimetry according to Bartlett (*14*). Lipids of pools of VLDL, HDL1, and HDL2 fractions were extracted with chloroform : methanol (2 : 1 v/v) according to Folch *et al.* (*15*). Cholesteryl ester (CE), TG, and PL were isolated from the lipid extracts by thin-layer chromatography (*16*). Each lipid classes was analyzed for its fatty acid distribution by gas-liquid chromatography (*16*). Bile salts were determined by enzymatic assay with 3-α-hydroxysteroid dehydrogenase, as described by Turley and Dietschy (*17*). A drop of each dialyzed fraction was examined by transmission electron microscopy (TEM) after staining with sodium phosphotungstate (*18*). Observations were made at 50,000× magnification with a Zeiss EM 10C electron microscope operating at 60 kV. Particle size was assessed using micrographs in a field of 500-1,000 particles counted on a final print varying from 30,000× to 150,000× magnification.

RESULTS

Total protein, lipid, free cholesterol, cholesteryl ester, triacylglycerol, and phospholipid outputs from perfused livers of both adult and young in perfused medium donors are shown in Table I. The density intervals both protein and lipid content and the percent composition of the three

TABLE I. **Total protein and lipid output from perfused livers from adult and young rat donors.** Levels of free and esterified cholesterol, triacylglycerol, and phospholipid are also reported. Data are means±SD of five experiments each group and are expressed in μg/g liv/2 h.

	Adult	Young
Protein	132.0±43.0	152.0±37.0
Lipid	271.0±23.0	277.0±29.0
FCH[a]	13.0±1.6	11.6± 1.3
CE[b]	23.2±2.5	22.6± 3.1
TG[c]	61.4±19.3**	104.9±14.3
PL[d]	94.1±4.6**	60.9± 5.1

**Adult *vs.* young group: $p<0.001$. [a]FCH, free cholesterol; [b]CE, cholesteryl esters; [c]TG, triacylglycerols; [d]PL, phospholipids.

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TABLE II **Lipoprotein fractions secreted from perfused livers of adult (14±1 month old) and young (3.5±0.5 month old) rat donors.** For each fraction it is reported the density interval used for their isolation from perfusion medium, the lipoprotein output and the percent distribution of the lipoprotein components is also reported. The value reported are means±SD of five perfusion experiments for each group.

		VLDL		HDL1		HDL2	
		Adult	Young	Adult	Young	Adult	Young
Output (μg/g liv/2 h)	Protein	8.6± 2.5	12.7± 6.1	27.3±5.7*	44.4±15.1	96.0±33.1	95.0±22.1
	Lipid	70.9±25.9*	145.0±52.7	65.5±7.6	70.0±19.0	135.0±10.0**	62.0±17.1
Percent composition	Protein	9.5± 3.9	8.1± 2.7	28.4±4.6*	38.8± 8.8	41.5±11.3	60.1±15.0
	FCH[a]	3.4± 2.2	3.8± 1.9	8.1±1.9	5.5± 2.7	3.9± 1.7	3.6± 2.9
	CE[b]	5.3± 2.7	4.6± 2.1	13.0±2.6*	20.5± 4.1	6.5± 2.4	2.8± 1.3
	TG[c]	52.0± 6.2	61.4± 8.0	18.4±6.5	14.0± 5.1	9.1± 2.9	9.8± 2.6
	PL[d]	29.8± 4.9*	22.1± 5.1	32.1±3.7**	21.2± 3.9	39.0± 4.8**	23.7± 7.1

[a]FCH, free cholesterol; [b]CE, cholesteryl esters; [c]TG, triacylglycerols, [d]PL, phospholipids. *Adult *vs* young group $p<0.05$. **Adult *vs.* young group: $p<0.01$.

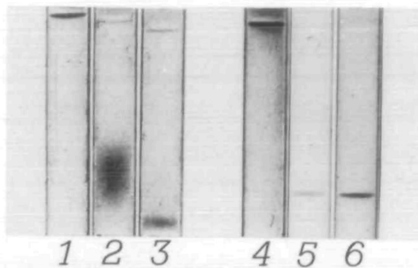

Fig 1. **Native PAGE of hepatic lipoproteins.** Each lipoprotein fraction, secreted in perfusion medium from livers of adult (gel 1 to 3) and young (gel 4 to 6) rats, was prestained with (the lipid stain) Sudan B black and analyzed in native 3.5% non denaturating polyacrylamide gel tube electrophoresis according to Naito *et al.* (*11*). Lanes 1 and 4: VLDL, lanes 2 and 5: HDL1, lanes 3 and 6: HDL2.

lipoprotein fractions analyzed are given in Table II. The fourth fraction at higher density ($d>1.022$ g/ml) contained mostly phospholipids and protein (about 80 and 15%, respectively). Albumin represented the main component of this fraction, but also Apo AI, and apolipoproteins C were detectable (data not shown). This fraction did not undergo further analyses.

The electrophoretic profile lipoprotein fractions in native PAGE (3.5% polyacrylamide) is reported in Fig. 1. The fraction with density below 1.04 g/ml showed the characteristics of VLDL class (lanes 1 and 4 for adult and young group, respectively). The fractions with density ranges between 1.05-1.09 and 1.10-1.21 g/ml showed electrophoretic mobility characteristics of HDL classes. They were identified as HDL1 and HDL2 fractions, respectively. HDL1 secreted by the livers of older donors (Fig. 1, lane 2) migrated as a band broader than HDL1 secreted from younger donors (Fig. 1, lane 5). A difference appeared also in the migration of HDL2 when comparing adult and young donors livers (Fig.1, lanes 3 and 6, respectively).

The total protein secretion and the total lipid secretion in the lipoprotein fractions analyzed did not show significant differences (Table I). Triacylglycerol output was significantly lower with livers of adult than young donors ($n=5$, $p<0.001$). The two groups showed a similar total output of cholesteryl ester into the perfusion medium, but the proportion of cholesteryl ester secreted by adult livers in HDL1 (density range 1.05-1.09 g/ml) was significantly lower in adult group with respect to young rats (Table II). Total phospholipid secretion was significantly higher in

TABLE III. **Composition characteristics of lipoproteins isolated from perfusion media of young and adult livers as evaluated by the molar ratios between the major lipid components.** Each value represents the mean±SD of five perfusion experiments.

		TG/PL	TG/CE	TG+CE/PL
Adult	VLDL	1.5±0.7*	7.2±2.8	1.7±0.9
	HDL1	0.5±0.2	1.5±0.7**	0.9±0.5
	HDL2	0.2±0.1	1.5±1.1	0.3±0.2
Young	VLDL	2.4±0.2	9.7±3.7	2.6±0.7
	HDL1	0.7±0.2	0.5±0.2	1.6±0.5
	HDL2	0.4±0.2	2.3±1.3	0.6±0.4

*Adult *vs.* young: $p<0.05$. **Adult *vs.* young: $p<0.01$.

livers from adult than from young rats ($n=5$, $p<0.001$). This difference was reflected by the significantly higher proportion of phospholipid found in each lipoprotein fraction secreted by adult livers (Table II). Moreover, HDL1 showed a significantly different protein content being HDL1 particles of adult were in fact, depleted in protein with respect to particles from young group (Table II).

In adult animals, VLDL, HDL1, and HDL2 fractions contained about 26, 24, and 50% of total lipid secreted, respectively. In young rats, the same fractions contained about 53, 26, and 23% of total lipids, respectively. These data, derived from results reported in Table I, indicate that adult liver secretes lipids mostly in HDL2 fraction and young liver mostly in VLDL. The lipid secretion associated with the HDL2 fraction was significantly higher in livers from older than younger donors. Age-related differences also appeared in the values of molar ratios between core to surface lipids (TG/PL and TG+CE/PL) and of TG/CE that are reported in Table II. These values were higher or equal in all lipoprotein fractions secreted by livers from younger animals with the exception of TG/CE ratio in HDL1 particles. This value was significantly higher in HDL1 from adult livers (1.5+0.7 and 0.5+0.2, $n=5$, $p<0.01$).

The apoproteins secreted by the two groups of donors did not show any significant differences in their percent distribution. Apo AI was the main apolipoprotein in HDL1 and HDL2 (about 90 and 65%, respectively). HDL1 contained traces of other apoproteins, *e.g.*, Apo AIV and Apo E. Apart from Apo AI, the HDL2 fraction contained appreciable amounts of Apo C's (variation from 20 to 30%).

Figure 3 shows the mean diameters of lipoprotein particles evaluated by frequency analysis from TEM micrographs of particles secreted in perfusion medium.

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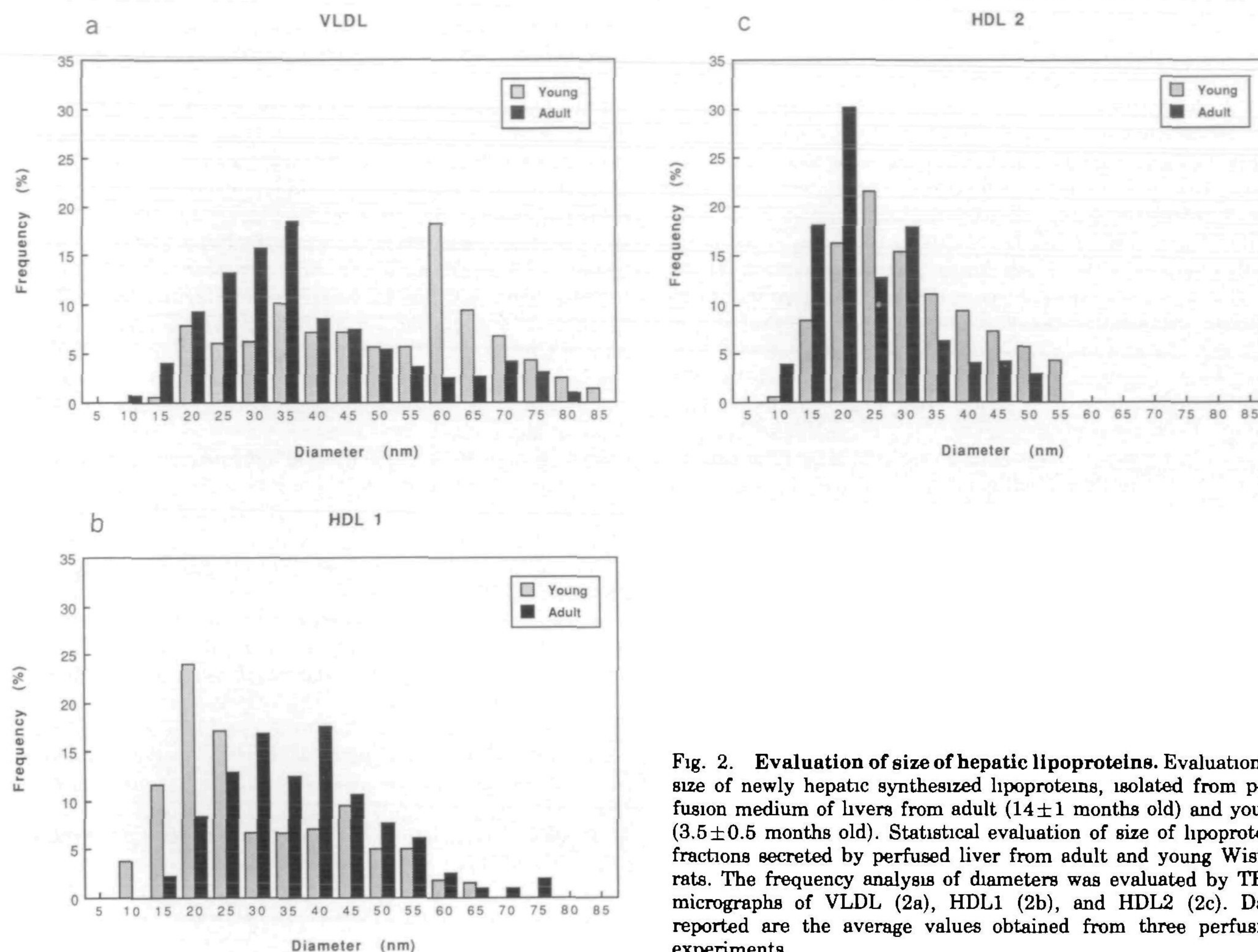

Fig. 2. **Evaluation of size of hepatic lipoproteins.** Evaluation of size of newly hepatic synthesized lipoproteins, isolated from perfusion medium of livers from adult (14±1 months old) and young (3.5±0.5 months old). Statistical evaluation of size of lipoprotein fractions secreted by perfused liver from adult and young Wistar rats. The frequency analysis of diameters was evaluated by TEM micrographs of VLDL (2a), HDL1 (2b), and HDL2 (2c). Data reported are the average values obtained from three perfusion experiments.

TABLE IV **Bile flow, lipid concentration, secretion and composition in biles collected during perfusion experiments with adult (14±1 month old) and young (3.5±0.5 month old) rat livers.** Results are means±SD of five experiments

	Bile flow (μl/h/g liv)	Biliary lipid concentration (μmol/ml)			Biliary lipid output (nmol/g liv/2 h)			Molar percentage (%)		
		Cho[a]	PL[b]	BS[c]	Cho[a]	PL[b]	BS[c]	Cho[a]	PL[b]	BS[c]
Adult	19 3±2.7**	0.33±0 11	1.92±0.20**	2.92±0.89	6.4±1.8	47.8±19.1*	106±32	4.1±0 9	30.8±12 6*	65 1±1.0
Young	26.5±3 9	0.29±0 07	0.76±0 22	2.87±1.10	7 0±2.1	24.1±9.9	134±55	4 7±1.9	15.6±3.6	79.7±9 7*

[a]Cho, cholesterol; [b]PL, phospholipids, [c]BS, bile salts *Adult *vs.* young group $p<0\ 05$. **Adult *vs.* young group: $p<0\ 001$.

Panels a, b, and c show the distribution of diameters in VLDL, HDL1, and HDL2 fractions, respectively.

Bile flow and lipid secretion data reported in Table IV show that adult livers secreted less bile than young livers (19.3±2.7 and 26.5±3.9 μl/h/g liver, adult and young rats respectively; $n=5$, $p<0.01$). However, bile secreted by adult livers had a significantly higher concentration, molar percentage and total output of phospholipids than bile secreted by young livers. Biliary steroid secretion did not show significant age-related differences, but the molar percentage of bile salt was lower in adult group (Table IV). The cholesterol to phospholipid molar ratio in microsomal membranes did not show statistically significant age-related differences (0.148±0.26 in adult livers and 0.137±0.012 in young livers).

DISCUSSION

Our previous studies with Wistar rats showed that levels of cholesterol, both circulating and stored in liver cells, increased with the aging process (*1, 2*). In order to clarify the role of hepatic lipid secretion in this age-related alteration of cholesterol equilibria, we studied lipid and lipoprotein secretion in perfused livers of both aged and young rats. The present data suggest that the age of the liver quantitatively affects the phospholipid and triacylglycerol secretions more than the cholesterol output. In fact, age-related differences in liver lipid output observed can be attributed, for the most part, to a higher proportion of phospholipids in the lipoprotein fractions secreted by the livers of older rats.

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Phospholipid is a surface component known to strongly affect the physical-chemical properties of lipoproteins. Its increase, if not paralleled by an increase in core components, determines a decrease in lipoprotein particles size. We found that VLDL and HDL2 particles isolated from older donors showed a smaller average diameter (Fig. 2, a and c). This is furtherly supported by the fact that both molar ratios including the surface lipid components (*i.e.*, TG/CE and TG+CE/PL) of lipoprotein particles from adult livers showed values lower than young livers. However, HDL1 particles showed lower molar ratios values of core/surface components associated to particles that are, in average, bigger than those isolated from young livers (Fig. 2b). This apparent contrast may be explained by the different structure of particles secreted from the two groups. Both the protein content and the CE/TG value, that is the ratio between two core components of lipoprotein, were in fact significantly higher in HDL1 particles isolated from adult compared to younger liver donors (Table III).

Livers from adult rats showed a reduced secretion of VLDL particles that resulted enriched in phospholipid and cholesteryl esters and deprived in triacylglycerol. These differences were reflected by a significant decrease in TG/PL molar ratio values in VLDL secreted from livers with respect to those of young group (Table III) and a different size distribution of VLDL in the two groups (Fig. 3a). Young livers showed a clear bimodal distribution of VLDL characterized by the presence of a second larger component. This indicates that the major age-related difference in the VLDL secretion of rat is the strong decrease in these lipid-rich VLDL with a larger core. These changes in newly synthesized hepatic VLDL (*i.e.* enrichment of PL and CE with reduced TG) are consistent with our previously reported age-related modifications, in the composition of circulating VLDL in the same rat strain (*1, 2*). These findings are in agreement with results of Van Lenten and Roheim (*4*) who reported in old Wistar rats a reduced VLDL concentration and a higher plasma concentration of apoB, with a proportional enrichment in triacylglycerol in other lipoprotein fractions in comparison with younger animals. Moreover, they reported an age-related increase in the proportion of apo B with a lower molecular weight with respect to higher molecular weight forms of this apolipoprotein (*4*). On the whole, these findings suggest that aging process impairs the liver capability to secrete VLDL, or better a larger VLDL subpopulation particles.

The variations in newly synthesized HDL particles observed in this study with perfused liver do not help to explain the age-related changes in plasma HDL subclasses found *in vivo* (*1, 3, 4*). In particular, the lack of increase in particles of HDL1 range by adult livers suggests that the age-related increase in the proportion of HDL1 previously observed in rat plasma (*1, 3, 4*) does not depend on an increased liver secretion of HDL1. This is an indirect support to the hypothesis that the accumulation of HDL1 particles in plasma may depend on a reduced catabolism of HDL1 during aging. This is supported by another our recent study in which we found that [^{14}C]cholesterol carried by HDL1 is taken up more slowly by the liver of 13 month old than of 3.5 month old Wistar rat and is paralleled by a decreased biliary secretion of the label by cannulated livers from older animals (*19*).

The phospholipid increase in bile secreted by older livers, in terms of both concentration and total output, is apparent contrast with a 37% decrease in the bile flow of liver from older donors (Table IV). However, a higher secretion of biliary phospholipids has been previously reported in aged Wistar and Sprague-Dawley rats (*5, 20, 21*). Food restriction affected bile flow and biliary steroid composition in animals of different ages, but did not affect the secretion of biliary phospholipids observed in older rats (*20, 21*). Thus, the uncoupled secretion of phospholipids and bile acids seems to be a peculiarity of the aging process. It has been hypothesized that this may derive from formation of either a more detergent bile, or from misdirected secretion of phospholipids from the sinusoidal to the canalicular pole of the hepatocyte in older animal, as it might be expected if the microtubule network function were compromised (*22*). Against the former idea, the same authors found that the change in bile acid composition in old rats could not account for the increased output of phospholipid in bile (*5*). On the other hand, the present study shows that in the interval of age investigated also the sinusoidal phospholipid output (lipoproteins) is increased, excluding the possibility of a misdirected PL secretion.

In conclusion, liver secretion appears to be modified during aging mainly by changes observed in VLDL, HDL1, and HDL2 that consist in a decreased hepatic secretion of triacylglycerols and a more marked increase in secretion of phospholipid. These changes may be due to a reduced conversion of 1,2-diacylglycerol in triacylglycerol and a concomitant increased conversion of it into phospholipids (*23*). This might be put in connection with other alterations, described in aging, as charge of composition (*24*) and/or properties (*25, 26*) of liver membranes.

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